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First successful hybridization experiment between native European weatherfish (*Misgurnus fossilis*) and non-native Oriental weatherfish (*M. anguillicaudatus*) reveals no evidence for postzygotic barriers

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Abstract

The European weatherfish *Misgurnus fossilis* (Linnaeus, 1758) is a threatened freshwater species in large parts of Europe and might come under pressure from currently establishing exotic weatherfish species. Additional threats might arise if those species hybridize which has been questioned in previous research. Regarding the hybridization of *M. fossilis* × *M. anguillicaudatus* (Cantor, 1842), we demonstrate that despite the considerable genetic distance between parental species, the estimated long divergence time and different ploidy levels do not represent a postzygotic barrier for hybridization of the European and Oriental weatherfish. The paternal species can be easily differentiated based on external pigment patterns with hybrids showing intermediate patterns. No difference in standard metabolic rate, indicating a lack of hybrid vigour, renders predictions of potential threats to the European weatherfish from hybridization with the Oriental weatherfish difficult. Therefore, the genetic and physiological basis of invasiveness via hybridization remains elusive in *Misgurnus* species and requires further research. The existence of prezygotic reproductive isolation mechanisms and the fertility of F1 hybrids remains to be tested to predict the potential threats of globally invasive Oriental weatherfish species.

Keywords

Freshwater fish, genome size, pigment patterns, postzygotic barrier, standard metabolic rate

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Introduction

Freshwater fish biodiversity is declining at an alarming rate (Reid et al. 2019; Tickner et al. 2020). Among other factors, habitat loss and invasive alien species are major drivers of species extinction (Clavero and Garcia-Berthou 2005; Cucherousset and Olden 2011; Closs et al. 2016). Wetlands are disappearing worldwide due to low agricultural value, facilitating conversion to arable land or other land "developments". Fishes specifically adapted to such habitats and serving as indicator species for the ecological status of wetlands, e.g. mudminnows (*Umbra krameri*, Walbaum 1792) (Wanzenböck 2004), are particularly affected (Tickner et al. 2020). Especially if habitat loss is combined with threats from closely related invasive species, effects might be non-additive and massive (Clavero and Garcia-Berthou 2005; Didham et al. 2007). Such a scenario is currently developing for the European weatherfish (*Misgurnus fossilis*, Linnaeus 1758) and the closely related, invasive Oriental weatherfish (*Misgurnus anguillicaudatus* (Cantor, 1842)).

The European weatherfish is a native species distributed across Europe and western Asia and is of significant conservation concern (Bohlen et al. 2007; Freyhof 2013). It is included in the list of freshwater fish species requiring international protection (European Union Habitats Directive and Bern Convention, see https://eunis.eea.europa.eu/species/551#legal_status) and on a number of national red lists (Wolfram and Mikschi 2007). Declining populations are primarily based on habitat loss (Wolfram and Mikschi 2007; Brys et al. 2020b) because the European weatherfish is specifically adapted to floodplain backwaters and wetlands of large rivers (Ràb et al. 2007). However, the invasion by the closely related Oriental weatherfish might contribute to the disappearance of European weatherfish in the future (Riffel et al. 1994). The Oriental weatherfish was imported as early as 1870 to Hawaii by Asian immigrants using it as food fish (Nico et al. 2019), and to the rest of the USA in the late 19th century by the ornamental pet fish industry as an aquarium and garden pond fish (Maceda-Veiga et al. 2013; Nico et al. 2019). Established populations in the wild have been found in many parts of the world (Milton et al. 2018) including Australia, Europe, South America (Abilhoa et al. 2013) and the USA (Frable 2008; Nico et al. 2019). In Europe, feral exotic weatherfish were initially found in Germany by Riffel et al. (1994) reporting, besides their genetic study on fish from a pond, a wild individual of *M. mizolepis* (Günther) being caught in the Nahe river (Rhineland-Palatinate) in 1993. Subsequently, Oriental weatherfish were recorded in Italy (Razzetti et al. 2001), Germany (Freyhof and Korte 2005), Spain (Franch et al. 2008), the Netherlands (van Kessel et al. 2013), from where it presently expands into Belgium, (Verreycken pers. comm), to Southern Germany (Belle et al. 2017), and recently to Austria (Zangl et al. 2020). However, there is some discrepancy regarding the taxonomy of different populations of exotic weatherfish found in Europe belonging to *M. anguillicaudatus*, *M. bipartitus* (Sauvage & Dabry de Thiersant, 1874), *Paramisgurnus dabryanus* (Dabry de Thiersant, 1872) or *M. mizolepis* (Günther, 1888) (Milton et al. 2018; Zangl et al. 2020; Belle et al. 2021).

A high probability of direct contact of the European and Oriental weatherfish in the same waterbody can be foreseen in the near future because of similar habitat preferences

(Meyer and Hinrichs 2000; Frable 2008; Brys et al. 2020a), however, predictions of potential consequences for the native species are difficult. Apart from competition based on broadly overlapping ecological niches, threats are predictable based on close taxonomic relationship, facilitating hybridization (Riffel et al. 1994; Rhymer and Simberloff 1996; Rhymer 2008). In the case of successful hybridization, one could anticipate high risks based on competitive advantages of hybrids (Huxel 1999; Rosenfield et al. 2004; Schierenbeck and Elstrand 2009; Coulter et al. 2020), especially if the hybrids show heterosis effects. On the other hand, risks from hybrids might be low if hybrids are not viable or are infertile representing a post-zygotic isolation mechanism (Janko et al. 2017).

Therefore, we tested in a primarily qualitative study for 1) the presence/absence of postzygotic reproductive isolation measured as embryo/larval viability by conducting a reciprocal hybridization experiment, and 2) the presence/absence of heterosis effects manifested in a physiological trait such as standard metabolic rate in relation to genome size and/or ploidy levels.

Material and methods

Fish propagation

Eight adult individuals of the European native species (European weatherfish, *M. fossilis*, see Suppl. material 1: Fig. S1, Suppl. material 2: Fig. S2) were obtained from a wild population in the floodplain of the River March (=Morava, close to Baumgarten) forming the border between Austria and Slovakia. Electrofishing was used on March 29, 2018, to catch adult fish intended primarily for artificial reproduction and restocking of juveniles within a conservation project. They were transferred to Mondsee, Austria, and held in a large aquarium (350 Liter) for 8 weeks. Eggs of one large (23 cm total length) female (out of 4 females) and sperm from 3 adult males (out of 4 males) were used for the hybridization experiments.

Five adult fish of the non-native species *M. anguillicaudatus*, (see Suppl. material 3: Fig. S3, Suppl. material 4: Fig. S4) were obtained from a garden pond population in summer 2017. 10 morphometric parameters following Yi et al. (2017), identified them as *M. anguillicaudatus* and discriminated them from *M. bipartitus*, another exotic weatherfish species found in Europe (Zangl et al. 2020). The most discriminating ratio values (i.e. caudal peduncle length/body length, caudal peduncle height/caudal peduncle length) were found to be 0.163±0.015 (mean±SD) and 0.619±0.085, respectively, and were always closer to the values given for *M. anguillicaudatus* than to *M. bipartitus* (table 1 in Yi et al. 2017). A misidentification with *P. dabryanus* can be excluded due to our genome size measurements which show a C-value of approx. 1.65 pg/nucleus. According to "Animal genome database" (T. Ryan Gregory https://www.genomesize.com/results.php?page=1) diploid *M. anguillicatudatus* are in the range of 1.37–1.86pg/nucleus. *P. dabryanus* has significantly lower values in the range of 1.07–1.11 pg/nucleus. Furthermore, misi-

dentification with *M. bipartitus* can be excluded based on genetic identity analysis of the mitochondrial 12S marker according to Miya et al. (2015). Our individuals have been sequenced using MiFish primers and showed percent identity values of 96.55–99.43 to *M. anguillicaudatus* sequences published in NCBI, whereas they showed values of only 93.6% to *M. bipartitus*. Percent identity values to *P. dabryanus* showed values of 86.71–87.86.

Non-native fishes were held in a large (400 Liter), unheated aquarium over the autumn 2017 and winter 2017/2018. Eggs of two females (18 and 22 cm total length respectively) and sperm of 3 adult males were used for the experiments. The aquarium was in an unheated room and was exposed to natural temperature rise in spring 2018, and natural daylight from a large window in close proximity. Three weeks before hormone treatment was initiated (May 25), both aquaria inhabited by native and alien weatherfish respectively, were heated using aquarium heaters from 16 °C to 21 °C at a rate of 1 °C every other day.

Hormone treatment was performed following Kouril et al. (1996) and Schreiber et al. (2017) using Ovopel (http://ovopel.hu/en/). Brood fish were injected by Ovopel solutions into the dorsal muscle according to manufacturer's instructions (1 pellet per kg body weight) and gametes were stripped 48 hours later (May 27). Eggs were artificially inseminated in all four reciprocal mating combinations, i.e. eggs of one European weatherfish female (approx. 4,000) were divided in half and one portion was inseminated with mixed sperm of three European weatherfish males, the second portion with mixed sperm of three Oriental weatherfish males. Similarly, mixed eggs of two Oriental weatherfish females (approx. 4,000) were divided in half and one portion fertilized with mixed sperm of three Oriental weatherfish males, the second portion with mixed sperm of three European weatherfish males. Fertilized eggs were incubated in jars which were placed in a 60 L aquarium (21 °C) and aerated. Larvae hatched the next day (May 28). Unfortunately, we obtained only very few hybrid larvae of the mating between female *M. anguillicaudatus* and male *M. fossilis* due to accidental clogging of the incubation jar causing detrimental oxygen shortage. The groups of larvae were kept in separate 30 L aquaria each, containing lake water at a temperature 24 °C ± 2 °C, supplied with artificial light (day: night 12 h:12 h). The few hybrid larvae produced from Oriental weatherfish females and European weatherfish males (estimated 5–10 individuals) disappeared during the following three days – the last larva observed was killed by a large copepod introduced accidentally into the aquarium, thus excluding this group from further analysis. Eggs (estimated to be 1,000–2,000 per group) and larvae developed normally in the other three groups, similar to previous artificial reproduction campaigns of *M. fossilis*, photographically documented in Schauer et al. (2013). Animals were fed ad libitum with dry feed, living *Artemia nauplii* (daily), and chopped worms (*Tubifex*) (once a week). In those groups, we obtained several hundred (>500) hatched larvae resulting in 200–500 juveniles each in autumn 2018. Exact quantification of survival rates in eggs and larvae/juveniles was not performed, and sex determination is not possible before maturity is reached (2 years).

Standard metabolic rate (SMR)

The Standard Metabolic Rate (SMR) is defined as the minimal amount of oxygen needed to maintain the aerobic metabolism. There are eight different definitions on how to estimate the SMR (Chabot et al. 2016). A time and cost-effective way, and one of the most common estimates, is to take the average of the 10% lowest oxygen consumption values (one value for each 15 min. measuring cycle) during the measurement period (36 hours) after removing the five lowest ones as outliers (low 10%). This analysis was used here as follows: Three acrylic respirometer chambers with a volume of 133 ml each were submerged in a basin filled with 25 L of aerated and treated (sand filter, UV disinfection) lake water (100% oxygen saturation; temperature 20 °C). Water temperature was controlled by a heating (Lauda "Alpha", Lauda, Regensburg, Germany) and a cooling unit (Lauda chiller). A Cyclobios respirometer controller was used to trigger the measuring intervals and flushing periods. Each measurement and flush interval was set to 15 min. Each cycle included three measurement intervals (one for each chamber) followed by one flush interval for all chambers simultaneously. One pump was used to flush the chambers, a second one pumped water from one chamber to an optical Oxygen sensor (YSI-Pro ODo, ecoTech, Bonn, Germany) during the measuring period and back to the chamber in a closed loop. The flow rate was 400 ml/min. In all experiments, fish were starved for 24 h before introducing them into the chamber. One chamber was left empty to determine bacterial oxygen consumption as baseline control. Before measurements, fish were acclimatized in the chamber for 12 hours. Each experiment lasted for 48 hours (including 12 hours of acclimation time) under the natural light regime. All individuals were juveniles and their wet weight ranged between 0.122 g and 1.98 g (mean: 0.65 g +/ – 0.416 g SD). If fish weight was less than one gram, measurements were conducted in groups of up to six individuals. For weights above one gram, the fish were measured individually to keep biomass in the chambers relatively constant following standard procedures (Chabot et al. 2016). The number of replicates (N in Figs 6 and 7) refers to single chambers and not individuals in the experiments. Statistical comparisons among groups (*M. fossilis*, *M. anguillicaudatus*, and hybrids) were done with SigmaPlot (Systat Software, Inc., version 14.0) applying Analysis of Covariance (ANCOVA) using average individual fresh weight as covariable.

Ploidy

Relative genome size was determined in 10 individuals of each group, following the protocol of Lamatsch et al. (2000). Isolated nuclei of fin clips (taken for identification purposes) from juveniles (6 months old) were stained with DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich, Vienna, Austria) and measured for fluorescence intensity with a violet laser (405 nm) in a flow cytometer with acoustic focusing technology (Attune NxT, ThermoFisher Scientific, Vienna, Austria) using chicken red blood cells as internal standard (2.5 pg per nucleus; Vinogradov 1998). Ploidy levels were assessed in comparison to relative genome sizes given in Drozd et al. (2010).

Figure 1. Pictures of weatherfishes taken one month after hatching (30.6.18) when larvae transformed into juveniles. Top panel: *M. fossilis* with larval fin fold still present. Center panel: Hybrid weatherfish with rests of larval fin fold present. Lower panel: *M. anguillicaudatus* – only small remnants of larval fin fold visible. Photographs: Sylvia Wanzenböck.

Results

Larvae developed normally without any apparent morphological aberrations in all three groups (*M. fossilis*, *M. anguillicaudatus*, and hybrids produced from *M. fossilis* females and *M. anguillicaudatus* males). Different pigmentation patterns for these two parental species became apparent during the first weeks of development, with hybrids showing intermediate pigmentation (Figs 1–4). Development to juveniles, judged from the disappearance of the larval fin-fold, occurred approximately 3 weeks later in *M. fossilis* compared to *M. anguillicaudatus* (when 50% individuals in the groups passed this stage) and was intermediate in hybrids.

The lateral side of *M. fossils* can be separated into 4 distinguishable color bands. The overall dorsal part is dark brown. The first band is light yellowish colored and clearly separates from the dark brown band along the lateral line. Ventral to the brown lateral line band another light yellowish color band follows. A thin but distinct dark brown color band separates the ventral side (brown to yellow or sometimes a slightly red color) from the second light yellowish color band. In all dark color bands, several small dark spots might be present. The dorsal dark brown color line on the lateral side can be dispersed into a loose line of brown dots and may end just below the dorsal fin. The most ventral brown color band extends from the basis of the pectoral fins to

Figure 2. Pictures of weatherfishes taken two months after hatching (21.7.18) when development to juveniles was nearly completed. Top panel: *M. fossilis* with larval fin fold still visible. Center panel: Hybrid weatherfish without larval fin fold. Lower panel: *M. anguillicaudatus* – juvenile and no larval fin fold visible. Photographs: Sylvia Wanzenböck.

the pelvic fins, and in some individuals to the basis of the anal fin. This band can be dispersed into a loose line or an interrupted row of brown dots.

The hybrids of *M. fossils* and *M. anguillicaudatus* show a brown-yellowish speckled overall pattern on the dorsal (and part of the lateral) side. The edge of this pattern often consists of a more or less continuous alignment of dark brown dots. It stretches from the eye to the base of the caudal peduncle. Often there is a loose and interrupted row of dots between the base of the dorsal fin and the caudal peduncle. The first color band is light yellowish and filled with several small dark brown dots. It stretches from the gill openings to the base of the caudal peduncle and contains various numbers of small brown dots, which increase in number with age. This color band is not as distinct as in *M. fossilis*. A dark brown color band along the lateral line from the gill openings to the base of the caudal peduncle forms the second color band. This one is also not as clearly distinct as in *M. fossilis*. The third light yellowish band, just next to the second one, is often filled with fine brown dots, and stretches from the base of the pectoralis to the base of the caudal peduncle. This more or less strongly dotted yellowish line is sometimes not clearly distinguishable from the fourth color band which is often dispersed into an interrupted and incomplete alignment of big brown dots. It extends from the basis of the pectoral fin to the ventral fin and in some individuals until the basis of the anal fin.

Figure 3. Pictures of weatherfishes taken three months after hatching (26.8.18) when development to juveniles was completed. Top panel: *M. fossilis*. Center panel: Hybrid weatherfish. Lower panel: *M. anguillicaudatus*. Photographs: Lukas Fuxjäger.

The basic coloration of *M. anguillicaudatus* varies from gray to sand-colored. On the dorsal and lateral side of the body more or less clearly visible dark dots are recognizable in an irregular pattern. Individuals with a high number of dark dots on the lateral side show a thin light band along the lateral line from the gill openings to the base of the caudal peduncle. Just before the dark bow on the very end of the caudal peduncle (sometimes divided, forming a dorsal and ventral black dot), a light half-moon shaped line is located. But there are also individuals without any dots. Pigmentation patterns were variable between individuals (Fig. 5).

Standard metabolic rate (SMR) was found to depend negatively on fresh weight even when oxygen consumption values were calculated per unit weight (gram fresh weight – Fig. 6). Nevertheless, regressions of groups (equal slopes, Fig. 6) and average values (Fig. 7) were statistically not significantly different between parental species and hybrids (ANCOVA, fresh weight as covariable, $p = 0.667$)

Measurements of ploidy levels revealed relative genome sizes of 5.20 (±0.123) pg/nucleus, 4.77 (± 0.07), and 3.33 (±0.03) on average for *M. fossilis*, M. hybrids and *M. anguillicaudatus*, respectively (Fig. 8). Differences between groups were found to be statistically significant (ANOVA, $p < 0.001$), and pairwise comparisons (Holm-Sidak method) revealed significant differences among all group pairs ($p < 0.001$). Since these measure-

Figure 4. Pictures of weatherfishes taken four months after hatching (1.10.18) showing advanced juvenile stages. Top panel: *M. fossilis*. Center panel: Hybrid weatherfish. Lower panel: *M. anguillicaudatus*. Photographs: Sylvia Wanzenböck.

ments are consistent with genome size estimates and chromosome counts of Drozd et al. (2010, see discussion), we categorized *M. anguillicaudatus* as diploid (2n), and *M. fossilis* as tetraploid (4n). The hybrids, however, showed an average genome size of 4.77 (± 0.07) pg/nucleus which is 0.5 pg higher than the expected average between the two parental species.

Discussion

Based on a genetic study using enzyme electrophoresis, Riffel et al. (1994) assumed that hybridization between European and Asian weatherfish might be hampered due to their genetic distance (Nei) of 0.9651, and a difference in ploidy level. The relatively high estimated divergence time between the two species (15–25.5 MYA; www.timetree.org; Tang et al. 2008) supports this hypothesis. We found, however, no postzygotic barrier in embryo survival or larval viability for interspecific F1 hybrids. Whether hybridization is qualitatively possible in both directions (*M. fossilis* females and *M. anguillicaudatus* males and vice versa) remains to be studied. However, based on our observations and hybridization studies in the weatherfish group (e.g. *M. anguillicaudatus* × *P. dabryanus*,

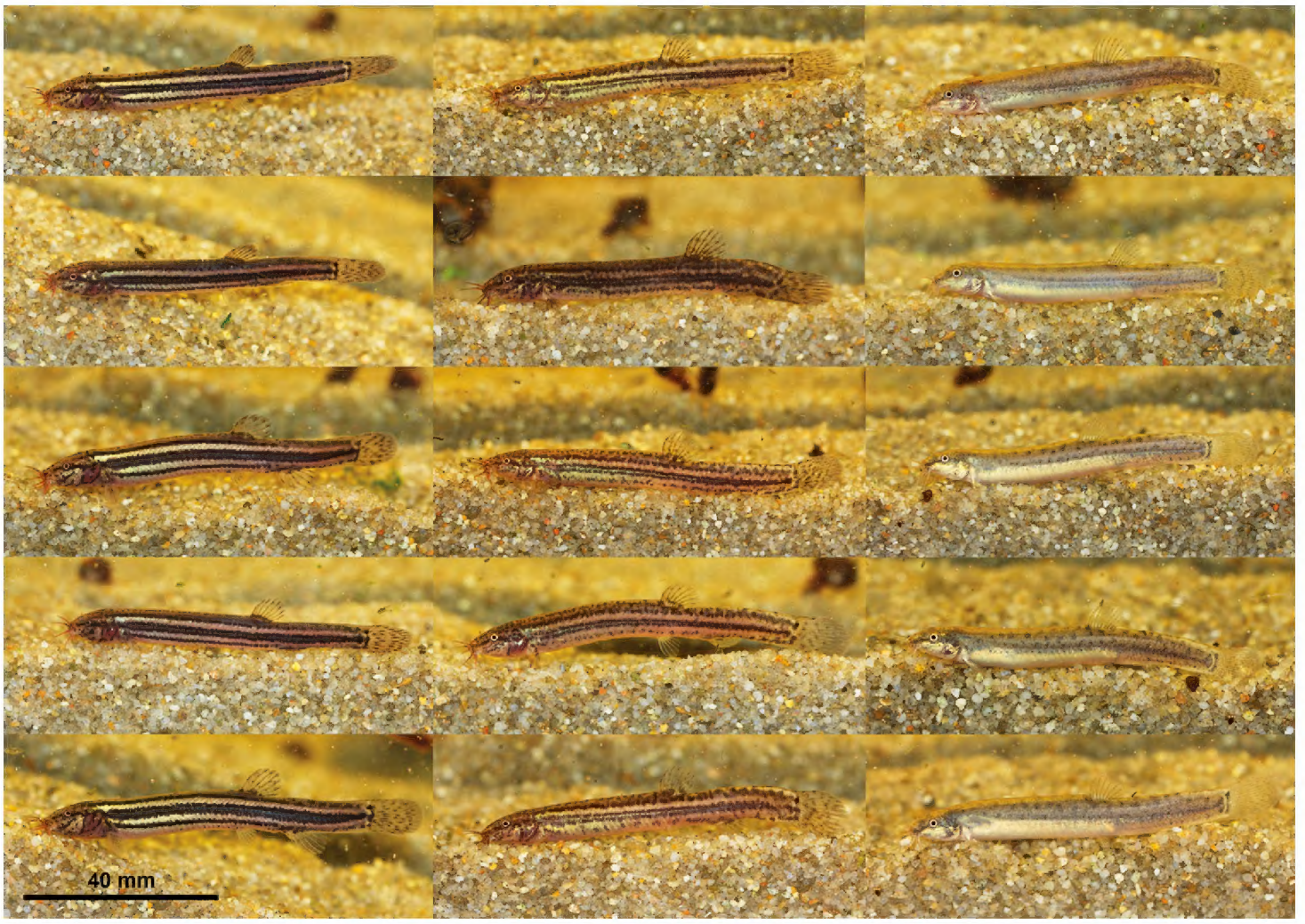

Figure 5. Pictures of weatherfishes taken four months after hatching (1.10.18) showing advanced juvenile stages and individual variation in pigmentation patterns. Left column: *M. fossilis*. Center column: Hybrid weatherfish. Right column: *M. anguillicaudatus*. Photographs: Sylvia Wanzenböck.

Huang et al. 2017) it seems very likely, given that the low survival of eggs and larvae of the hybrids produced from *M. anguillicaudatus* females and *M. fossilis* males could be linked to accidental events. However, we cannot yet determine if hybridization probability and hybrid viability are equal in both directions, or if embryonic and larval mortality is quantitatively asymmetric. Larval development in hybrid individuals was basically similar to pure species and successful. However, there was an indication of elevated malformations in hybrids (personal observation). Determining if hybrids attain sexual maturity and are fertile would be extremely important to assess the potential for hybrid swarms threatening wild populations of the native species (Rhymer 2008) and for our general understanding of the relationship between asexuality, hybridization, and speciation in fishes (Lamatsch and Stöck 2009; Janko et al. 2017).

Differentiation between *M. fossilis* and various exotic weatherfish (in our case *M. anguillicaudatus*) was found to be easily possible based on pigmentation patterns (see also Kottelat and Freyhof 2007). In concordance to other authors (e.g., Freyhof and Korte 2005; Kottelat and Freyhof 2007), we found that species identification is clear-cut based on longitudinal, characteristic and alternating dark and light pigment bands in *M. fossilis* which are absent in *M. anguillicaudatus* and other non-native species. We agree that the black dot in the dorsal part of the caudal base is an unsuitable characteris-

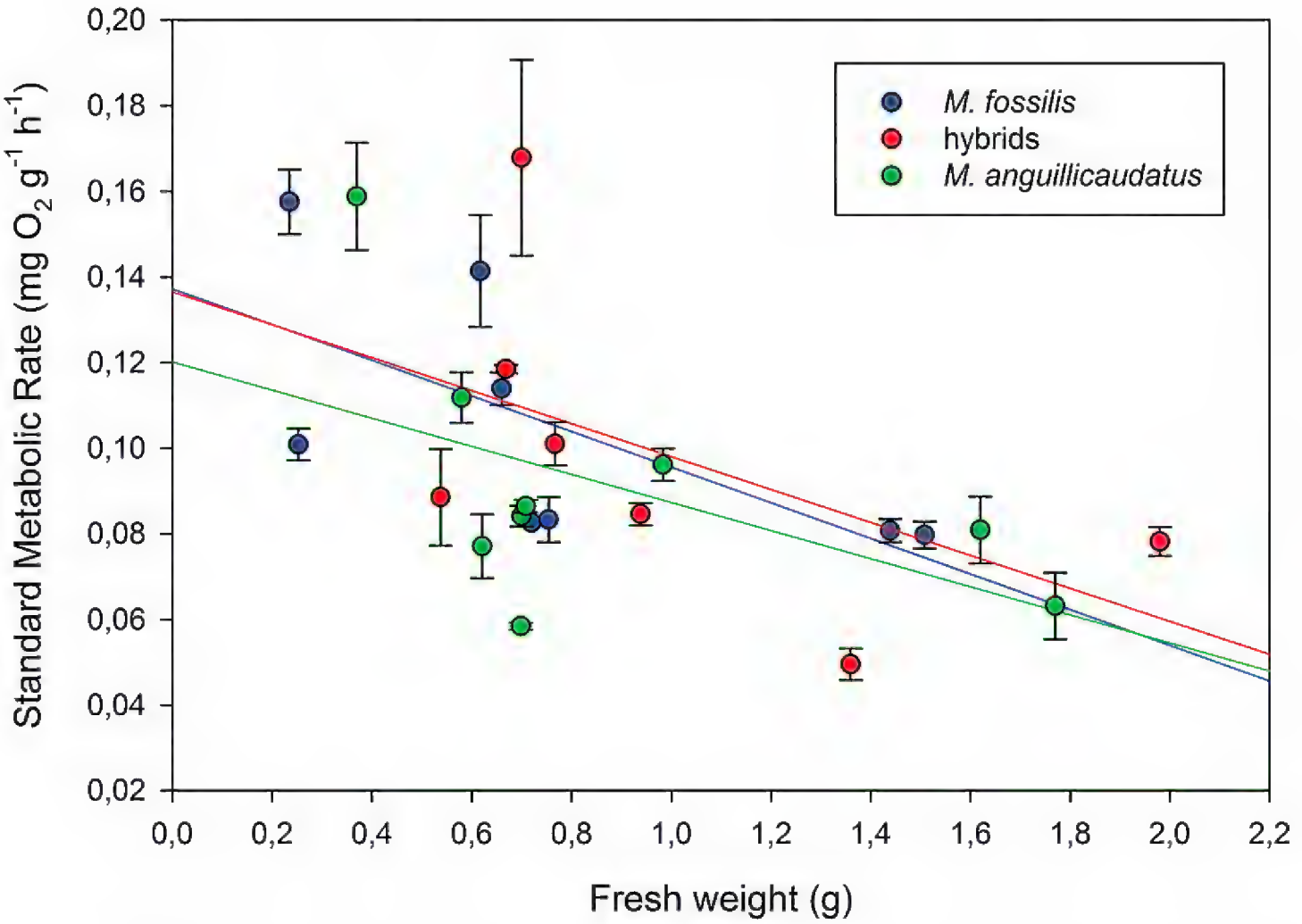

Figure 6. Standard metabolic rate (measured as oxygen uptake per gram fresh weight per hour of juvenile *M. fossilis* (N = 8), juvenile hybrids (N = 7) and juvenile *M. anguillicaudatus* (N = 9) versus fresh weight (average of lowest 10% measurements over time ± SD). Note that N in this figure refers to number of chambers.

tic for differentiation of *M. fossilis* vs. *M. anguillicaudatus*, as a black dot is also found in *M. fossilis*, at least in our juveniles (see Fig. 5). However, we want to stress that genetic methods, although reliable in *Misgurnus* (e.g., Miya et al. 2015; Yi et al. 2017; Belle et al. 2021) are not the only tool to correctly differentiate the native European weatherfish from introduced Oriental weatherfish, and identification should be possible by trained experts using morphological characters i.e., pigmentation patterns. Anyhow, the discrimination of each of the parental species versus their hybrids, solely on pigmentation patterns may seem difficult although it looked quite obvious to us (Fig. 5).

Our estimates of SMR, measured as oxygen consumption (VO2), were comparable to previous estimates in a closely related loach species, i.e. the spined loach, *Cobitis* sp. (Maciak et al. 2011). Our obtained average values of approximately 0.1 mg O_2 g^{-1} h^{-1} are higher compared to their values of approx. 0.05 mg g^{-1} h^{-1}. However, lowest values are similar (Fig. 6). Given the fact that VO2 values are generally highly variable and are mostly presented as logarithmically transformed values, we consider those values to correspond nicely. Our fish were too large and methods were too different for making meaningful comparisons with measurements on early larval European weatherfish by Schreiber et al. (2017), or those for Oriental weatherfish by White et al. (2017).

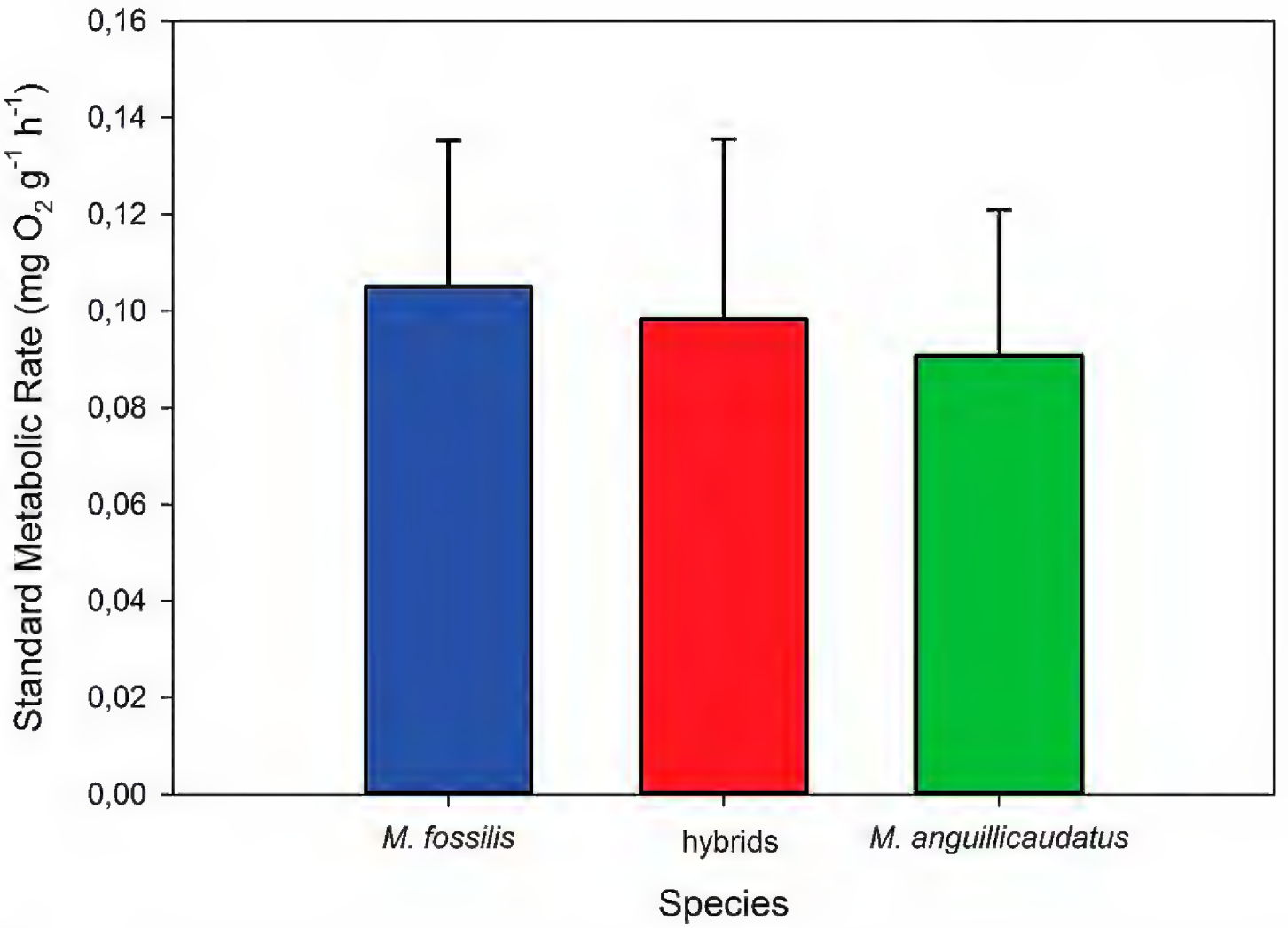

Figure 7. Average Standard metabolic rate (±SD) measured as oxygen uptake per gram fresh weight per hour for juvenile *M. fossilis* (N = 8), juvenile hybrids (N = 7) and juvenile *M. anguillicaudatus* (N = 9). Note that N in this figure refers to number of chambers.

We observed a negative trend in VO2 per gram wet mass with increasing body mass, which is a well – known pattern in the early ontogeny of larval and juvenile fish (Chabot et al. 2016) and reflects lower weight-specific metabolic rates with increasing body mass as the scaling exponent between oxygen consumption and mass is typically lower than one (Clarke and Johnston 1999; Isaac and Carbone 2010). The decreasing trends were similar for parental species and hybrids.

The relationship between metabolic rates and heterosis (or hybrid vigor, specifically in growth, i.e. "metabolic heterosis" *sensu* Ginn (2017)), does not show clear-cut trends: While decreased (i.e. more efficient) metabolic rates in hybrids are widely found in germinating plants (Sinha and Khanna 1975; Goff 2011), especially in crops, and could be expected based on molecular and cell physiological principles (Chen 2010, 2013; Goff 2011; Ginn 2017; Govindaraju 2019), the findings in animals are controversial. In marine bivalves Hedgecock et al. (1996) observed more efficient protein metabolism coupled with lower oxygen consumption in three out of four replicate experiments, whereas in geckos lower SMR was observed in hybrids in only one out of three regions (Kearney and Shine 2004). In fish, hybridization is widespread (Chevassus 1983; Scribner et al. 2000) and used mainly for aquaculture purposes (Wang et al. 2019). Lower metabolic rates and thus more efficient protein turnover rates were found in more het-

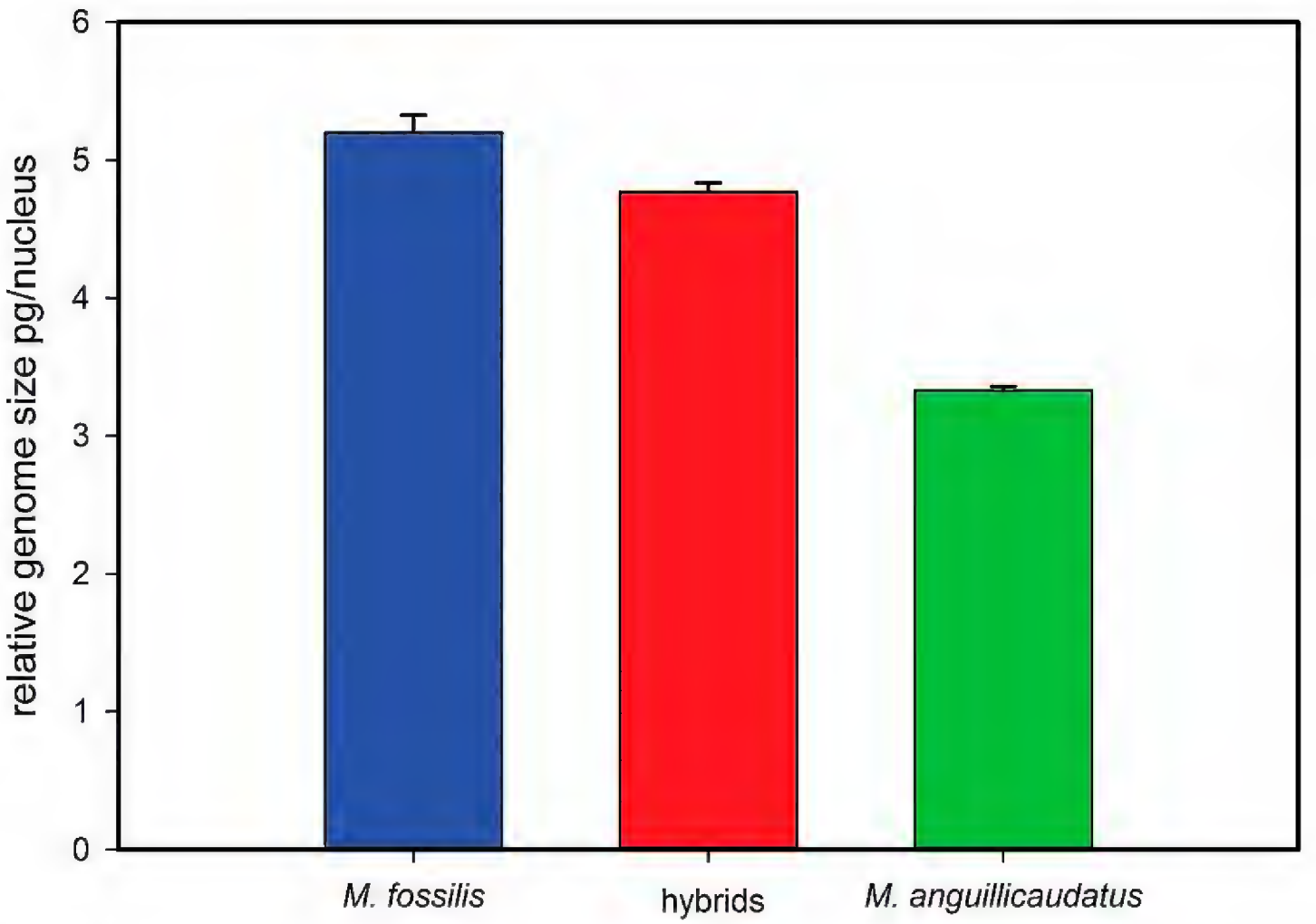

Figure 8. Relative genome sizes (DAPI stained) of individual fishes (N = 10 for each group) in pg/nucleus (average ± SD) relative to chicken red blood cells (2.5 pg/nucleus, Vinogradov 1998).

erotic individuals within a single strain of rainbow trout, i.e. intraspecific (Danzmann et al. 1988), and in hybrid bass compared to pure striped bass, i.e. interspecific (Tuncer et al. 1990). However, there are also many negative reports of hybrid strains showing no useful heterotic traits for breeding, e.g. chinook salmon (Bryden et al. 2004). In conclusion, the effect of heterosis and its relationship to physiological traits such as SMR in animals and specifically in fish remains elusive and is constrained by a low number of studies measuring those traits in intra- and interspecific hybrids. Therefore, we need more studies to test for genotype-phenotype relationships (Fiévet et al. 2018) involving not only the ultimate outcome such as growth and biological success (invasiveness) but also the underlying characters such as metabolic rates and physiological performance. Anyhow, there are several examples for interspecific hybrids showing heterotic, superior performance compared to parental species (e.g. in *Misgurnus*, Luo et al. 2021). Furthermore, there are cases where hybrids are threatening e.g. Pecos pupfish (Rosenfield et al. 2004) or showing superior invasiveness e.g., hybrid Chinese carps (Coulter et al. 2020), shiners (Blum et al. 2010), or bleak (Almodóvar et al. 2012).

Since representatives of the genus *Misgurnus* were found to show various ploidy levels (Ràb et al. 2007 and citations therein), correlating negatively with SMR (Maciak et al. 2011), ploidy needs to be considered to correctly interpret our results. *M. fossilis* from a population in the Czech Republic (158 km away from the population studied

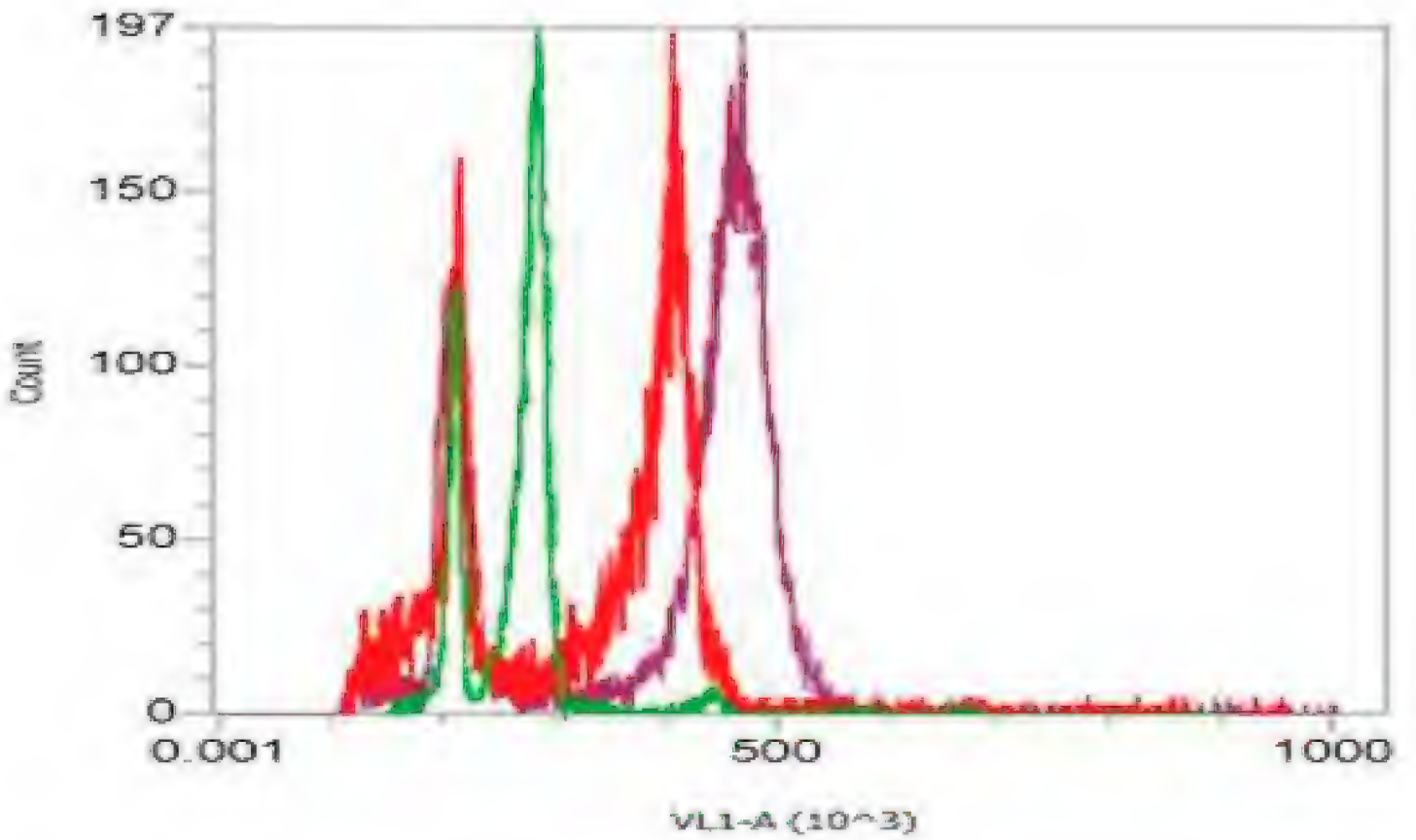

Figure 9. Example of flow cytometer measurements. The first peak from the left is the internal standard (chicken red blood cells; similar in all measurements). The following peaks represent *M. anguillicaudatus* (green), hybrids (red), *M. fossilis* (purple).

here, but from the Elbe drainage) were found to be dominated by tetraploid individuals (66%, Drozd et al. 2010). However, triploid and aneuploid individuals were also detected (17% each). Ploidy status was confirmed by karyotyping, and the c-values for diploid cells (gametes) were found to be 2.02 pg/nucleus, slightly lower than the estimate of 2.6 pg/nucleus (Timofeeva and Kafiani (1964), cited in Animal Genome Size data base from Gregory (2020)). However, the database value corresponds to our relative genome sizes of 5.2 pg/nucleus for somatic cells. Because of this correspondence, we categorized our *M. fossilis* individuals as tetraploids. C-values of diploid *M. anguillicaudatus* (2n = 50 chromosomes, e.g. Zhang and Arai 2003) range from 1.37 pg (Park and Chung 1985) or 1.4 pg (Hinegardner and Rosen 1972) to 1.86 pg (Suzuki 1992). The C-values of Drozd et al. (2010) for 1n cells of diploid *M. anguillicaudatus* (1.65) correspond well with our average value of 3.3 pg/nucleus for 2n cells. This confirms our classification of *M. anguillicaudatus* being diploid. Hybrids showed an intermediate relative genome size and are therefore presumably triploid (3n). However, their genome size was slightly higher than the mean value of the two parental species. This might be due to the already observed aneuploidy and/or microchromosomes which can only be resolved by karyotyping.

Higher metabolic rates were found to correlate negatively with ploidy level in another loach species, i.e. spined loach, by Maciak et al. (2011). Because of this, we expected lower metabolic rates in the tetraploid European weatherfish compared to the diploid Oriental weatherfish. However, this was not apparent in our dataset. On

the contrary, there was a slight, though insignificant, tendency of a direct relationship between ploidy level and metabolic rate, i.e. the diploid Oriental species had a slightly lower SMR compared to the tetraploid European species with triploid hybrids showing intermediate SMR values (Fig. 7 and Fig. 8). The definitive lack of lower metabolic rates in the European weatherfish might be interpreted as a hint towards the secondary rediploidization in *M. fossilis* (Spóz et al. 2017).

Conclusion

We showed that there is no postzygotic barrier for hybridization between the European and Oriental weatherfish despite the assumption given by Riffel et al. (1994) that genetic introgression might be hampered by the large genetic distance (D = 0.9651), and ploidy level differences of the parental species. The existence of prezygotic reproductive isolation mechanisms and fertility of F1 hybrids remains to be tested. A lack of observable heterosis effects (hybrid vigour) in standard metabolic rate (SMR) renders predictions of potential augmented threats to the European weatherfish from hybridization with an Oriental weatherfish difficult. Therefore, the genetic and physiological basis of invasiveness via hybridization remains elusive within the genus *Misgurnus* species and requires further research. However, the European weatherfish is most probably threatened by the globally invasive Oriental weatherfish through competition, even without hybridization. A fact that should be considered in managing and conserving natural freshwater fish biodiversity in Europe.

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Supplementary material 1

Figure S1

Authors: Josef Wanzenböck, Mathias Hopfinger, Sylvia Wanzenböck, Lukas Fuxjäger2, Hans Rund, Dunja K. Lamatsch

Data type: Jpg file

Explanation note: Female Misgurnus fossilis used in the crossing experiment.

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Link: https://doi.org/10.3897/neobiota.69.67708.suppl1

Supplementary material 2

Figure S2

Authors: Josef Wanzenböck, Mathias Hopfinger, Sylvia Wanzenböck, Lukas Fuxjäger2, Hans Rund, Dunja K. Lamatsch

Data type: Jpg file

Explanation note: Males of Misgurnus fossilis used in the crossing experiment.

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Link: https://doi.org/10.3897/neobiota.69.67708.suppl2

Supplementary material 3

Figure S3
Authors: Josef Wanzenböck, Mathias Hopfinger, Sylvia Wanzenböck, Lukas Fuxjäger2, Hans Rund, Dunja K. Lamatsch
Data type: Jpg file
Explanation note: Female Misgurnus anguillicaudatus used in the crossing experiment.
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Supplementary material 4

Figure S4
Authors: Josef Wanzenböck, Mathias Hopfinger, Sylvia Wanzenböck, Lukas Fuxjäger2, Hans Rund, Dunja K. Lamatsch
Data type: Jpg file
Explanation note: Male Misgurnus anguillicaudatus used in the crossing experiment.
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Link: https://doi.org/10.3897/neobiota.69.67708.suppl4